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UTILITY PATENT APPLICATION TRANSMITTAL (Only for new nonprovisional applications under 37 CFR 1.53(b))		Attorney Docket No	10690/101683	Total Pages	65
		First Named Inventor or Application Identifier			
		Berend HOUWEN			
		Express Mail Label No.	EM330345405US		

APPLICATION ELEMENTS See MPEP chapter 600 concerning utility patent application contents							
1	<input checked="" type="checkbox"/> Fee Transmittal Form (Submit an original, and a duplicate for fee processing)					6 <input type="checkbox"/> Microfiche Computer Program (Appendix)	
2	<input checked="" type="checkbox"/> Specification Total Pages 29					7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)	
	<ul style="list-style-type: none"> - Descriptive title of the Invention - Cross References to Related Applications - Statement Regarding Fed sponsored R & D - Reference to Microfiche Appendix - Background of the Invention - Brief Summary of the Invention - Brief Description of the Drawings <i>(if filed)</i> - Detailed Description - Claim(s) - Abstract of the Disclosure 					<ul style="list-style-type: none"> a. <input type="checkbox"/> Computer Readable Copy b. <input type="checkbox"/> Paper Copy (identical to computer copy) c. <input type="checkbox"/> Statement verifying identity of above copies 	
3.	<input checked="" type="checkbox"/> Drawing(s) (35 USC 113) [Total Sheets 7]					ACCOMPANYING APPLICATION PARTS	
4.	<input type="checkbox"/> Oath or Declaration [Total Pages 3]					8. <input checked="" type="checkbox"/> Assignment Papers (cover sheet & document(s))	
a.	<input checked="" type="checkbox"/> Newly executed (original or copy)					9. <input type="checkbox"/> 37 CFR 3.73(b) Statement <input type="checkbox"/> Power of Attorney (when there is an assignee)	
b.	<input type="checkbox"/> Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional, see Box 17, completed) <i>[Note Box 3 below]</i>					10. <input type="checkbox"/> English Translation Document (if applicable)	
	<ul style="list-style-type: none"> i. <input type="checkbox"/> DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 133(b). 					11. <input checked="" type="checkbox"/> Information Disclosure Statement (IDS)/PTO-1449 <input type="checkbox"/> Copies of IDS Citations	
5	<input type="checkbox"/> Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.					12. <input checked="" type="checkbox"/> Preliminary Amendment	
17.	If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:					13. <input checked="" type="checkbox"/> Return Receipt Postcard (MPEP 503) (Should be specifically itemized)	
	<input type="checkbox"/> Continuation <input type="checkbox"/> Divisional <input checked="" type="checkbox"/> Continuation-in-part (CIP)					14. <input type="checkbox"/> Small Entity <input type="checkbox"/> Statement filed in prior application Statement(s) <input type="checkbox"/> Status still proper and desired	
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FEE TRANSMITTAL

Note: Effective October 1, 1997.
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Complete if Known

Application Number	To be Assigned
Filing Date	Concurrently herewith
First Named Inventor	Berend HOUWEN
Group Art Unit	To be Assigned
Examiner Name	To be Assigned
Attorney Docket Number	10690/101683

METHOD OF PAYMENT (check one)

1. The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:

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3. ADDITIONAL FEES

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee	Small Entity Fee Code (\$)	Fee	Fee Description	Fee Paid
105	130	205	65		Surcharge - late filing fee or oath	
127	50	227	25		Surcharge - late provisional filing fee or cover sheet	
139	130	139	130		Non-English specification	
147	2,520	147	2,520		For filing a request for reexamination	
112	920*	112	920*		Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*		Requesting publication of SIR after Examiner action	
115	110	215	55		Extension for reply within first month	
116	400	216	200		Extension for reply within second month	
117	950	217	475		Extension for reply within third month	
118	1,510	218	755		Extension for reply within fourth month	
128	2,060	228	1,030		Extension for reply within fifth month	
119	310	219	155		Notice of Appeal	
120	310	220	155		Filing a brief in support of an appeal	
121	270	221	135		Request for oral hearing	
138	1,510	138	1,510		Petition to institute a public use proceeding	
140	110	240	55		Petition to revive - unavoidable	
141	1,320	241	660		Petition to revive - unintentional	
142	1,320	242	660		Utility issue fee (or reissue)	
143	450	243	225		Design issue fee	
144	670	244	335		Plant issue fee	
122	130	122	130		Petitions to the Commissioner	
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126	240	126	240		Submission of Information Disclosure Stmt	
581	40	581	40		Recording each patent assignment per property (times number of properties)	
146	790	246	395		Filing a submission after final rejection (37 CFR 1.129(a))	
149	790	249	395		For each additional invention to be examined (37 CFR 1.129(b))	
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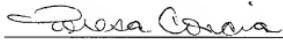
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Date: April 9, 1998


Teresa Concia

Teresa Concia

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:) Continuation-in-Part Application of Serial
Berend HOUWEN, *et al.*) No. 09/019,932, filed February 6, 1998
Serial No.: To be Assigned)) Examiner: To be Assigned
Filed: Concurrently herewith)) Art Unit: To be Assigned

For: PROCESS FOR DISCRIMINATING AND COUNTING ERYTHROBLASTS

New York, NY
April 9, 1998

Assistant Commissioner for Patents
BOX PATENT APPLICATION
Washington, D.C. 20231

CERTIFICATE OF EXPRESS MAILING

"Express Mail" mailing label number: EM330345405US

Date of Deposit: April 9, 1998

I hereby certify that the attached Continuation-in-Part Patent Application Transmittal PTO/SB/05, Free Transmittal Form PTO/SB/17, Application Cover Letter, 29 page Application (including 5 pages of claims and 1 abstract page), 7 sheets of Drawings, executed Declaration and Power of Attorney, Preliminary Amendment, Information Disclosure Statement, a check in the amount of \$790.00 to cover the filing fee for the application, Transmittal of Assignment and Submission of Document to Record Execution Date, Assignment with Recordation Cover Sheet, a check in the amount of \$40.00 to cover the Assignment fee, and this return postal card, are being deposited with the United States Postal Service "Express Mail Post Office To Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to Assistant Commissioner for Patents, BOX PATENT APPLICATION, Washington, D.C. 20231.

Teresa Concia

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April 9, 1998

Express Mail No. EM330345405US
Docket No. 10690/101683

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

Sir:

Pursuant to 37 C.F.R. §1.53(b), transmitted herewith for filing is the continuation-in-part application of Serial No. 09/019,932, filed on February 6, 1998 of

Inventor(s):

Berend HOUWEN;
Fu-sheng WANG;
Tomohiro TSUJI;
Takashi SAKATA; and
Yukio HAMAGUCHI,

For: PROCESS FOR DISCRIMINATING AND COUNTING ERYTHROBLASTS

Enclosed is:

1. The application transmitted herewith has 29 pages (including 5 pages of claims and 1 abstract page);
2. Seven (7) sheets of drawings are attached to the application;
3. A Declaration and Power of Attorney for Utility Patent Application - Joint;
4. A declaration and power of attorney:
 - a. [x] has been executed by all the inventors; or
 - b. [] has not been executed by all the inventors. A signed declaration and power of attorney will be submitted in due course;.
5. An associate power of attorney;

BRYAN CAVE LLP

Assistant Commissioner for Patents

April 9, 1998

Page 2

6. An assignment of the invention to TOA MEDICAL ELECTRONICS, CO., LTD., and a Recordation Form Cover sheet. Please record the Assignment and return it to the undersigned. A duplicate copy of this paper is enclosed;

a. A check for \$40.00 to cover the recording fee is enclosed. See paragraph 10.c., below.

b. Please charge the recording fee to our Deposit Account No. 02-4467. A duplicate copy of this paper is enclosed;

7. The Priority is hereby claimed under 35 USC §119 based on _____ Patent Appln. No. _____, filed on _____, 19____;

a. A certified copy of the priority document is enclosed.

b. The certified priority document(s) will follow;

8. A verified statement to establish small entity status under 37 CFR Section 1.9 and 37 CFR Section 1.27 is enclosed;

9. The filing fee is calculated below.

FOR:	Col. 1	Col. 2	SMALL ENTITY		OR	LARGE ENTITY	
	NO. FILED	NO. EXTRA	RATE	FEES		RATE	FEES
BASIC FEE				\$395.00	OR		\$790.00
TOTAL CLAIMS	12-20 =	*0	x 11.00	0.00	OR	x 22.00	0.00
INDEP. CLAIMS	1-3 -	*0	x 41.00	0.00	OR	x 82.00	0.00
MULTIPLE DEPENDENT CLAIM PRESEN/TLD			+ \$135.00	0.00	OR	- \$270.00	0.00
*If the difference in Col. 1 is less than zero, enter "-0" in Col 2							
Total: \$790.00							

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Assistant Commissioner for Patents

April 9, 1998

Page 3

12. A Preliminary Amendment;

- a. No additional fee is due;
- b. A check in the amount of \$____ to cover the cost of additional claims is enclosed. See paragraph 10.a., below;
- c. Please charge our Deposit Account 02-4467 in the amount of \$____. A duplicate copy of this paper is enclosed;

13. An Information Disclosure Statement;

The Commissioner is hereby authorized to charge payment of the following fees associated with this application or with recording any Assignment concerning it, or to credit any overpayment, to Deposit Account No. 02-4467, unless otherwise paid by check.

- a. If our check is missing or otherwise insufficient, or if any additional fees are required, the Commissioner is authorized to charge (or credit any overpayment) to Deposit Account No. 02-4467. **A duplicate copy of this paper is enclosed.**
- b. Any additional filing or other fees required under 37 CFR Section 1.16, including any fees for presentation of extra claims.
- c. Any patent application processing fees under 37 CFR Section 1.17.
- d. Any additional Assignment recording fees under 37 CFR Section 1.21(h).

Note: See 37 CFR §1.311(b) regarding authorization to pay the issue fee from deposit account.

Respectfully submitted,

By: Anne E. Zitron

Anne E. Zitron

Registration No. 41,391

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Date: April 9, 1998


Teresa Concia

Teresa Concia

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:) Continuation-in-Part Application of Serial
Berend HOUWEN, *et al.*) No. 09/019,932, filed February 6, 1998
Serial No.: To be Assigned)) Examiner: To be Assigned
Filed: Concurrently herewith)) Art Unit: To be Assigned

For: PROCESS FOR DISCRIMINATING AND COUNTING ERYTHROBLASTS

New York, NY
April 9, 1998

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend claims 3, 5, 8 and 9 as follows:

Claim 3, line 1, delete "or 2".

Claim 5, line 1, delete "any of claim 1 or 4" and insert - -claim 1- -.

Claim 8, line 1, delete "any of claims 1 to 7" and insert - -claim 1- -.

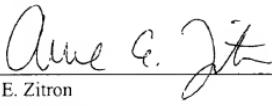
Claim 9, line 1, delete "any of claims 1 to 7" and insert - -claim 1- -.

REMARKS

The foregoing amendments were made to eliminate multiple dependent claims.

Respectfully submitted,

By: _____



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SPECIFICATION

PROCESS FOR DISCRIMINATING AND COUNTING ERYTHROBLASTS

BACKGROUND OF THE INVENTION

5 1. Field of the Invention

The present invention relates to a method for discriminating and counting erythroblasts. In particular, the present invention relates to a method for accurately discriminating and counting erythroblasts by flowcytometry.

10

2. Description of the Prior Art

The discrimination and counting of erythroblasts are of great use in a field of clinical examination since it provides very useful information for the diagnosis of diseases and prognosis of diseases.

15

Erythroblasts, which are also called nucleated red blood cells, are normally contained in the bone marrow, but not in peripheral blood except newborns. The appearance of erythroblasts in peripheral blood indicates a possible presence of a disease, such as leukemias, hemolytic anemia, iron deficiency anemia or pernicious anemia, and 20 other non-hematologic/oncologic disorders. Thus the discrimination and counting of erythroblasts are very effective for the diagnosis of any such disease and for the prognosis in some of them.

Conventionally, it has been usual to prepare a smear of blood, add an appropriate dye to the smear of blood and examine it by a 25 microscope for discriminating and counting erythroblasts. However,

such a method requires not only time-consuming, complicated pretreatment of blood for examination but also considerable expertise and skills for obtaining accurate results.

Recently, there have been available a variety of apparatuses
5 for the fully automated discrimination and counting of leukocytes based on the principle of a flowcytometer, and there have been proposed a number of methods for analyzing blood contents using such apparatuses.

For example, Japanese Unexamined Patent Publication No. HEI 4(1992)-268453 discloses a method for discriminating and
10 counting erythroblasts in a blood sample. This method includes the treatment of the blood sample with an acid reagent fluid of hypotonic osmolarity, the staining of nuclei of erythroblasts with a fluorescent dye solution and the detection of scattered light and fluorescent light by a flowcytometer.

Japanese Unexamined Patent Publication No. HEI 5(1993)-34251 discloses a method of determining erythroblasts. This method includes the treatment of a blood sample with an acid reagent fluid of hypotonic osmolarity, the staining of the blood sample with four kinds
20 of dyes including Astrazon Yellow 3G and Neutral Red which are fluorescent dyes and the detection of red fluorescent light and green fluorescent light by a flowcytometer.

Published Japanese translation of PCT international publication for patent application No. HEI 8(1996)-507147 discloses a
25 method for determining nucleated red blood cells by detecting forward

scattered light or fluorescence-side scattered light by a flowcytometer using a specific amount of a non-quaternary ammonium salt, an aliphatic aldehyde, a non-phosphate buffer, a reagent having a specific pH and a specific osmolarity and a nuclear dye such as ethidium

5 homodimer.

U.S. Patent No. 5,559,037 discloses a method for counting erythroblasts. This method includes the lysis of erythrocytes and cell membranes of erythroblasts, the staining with a vital nuclear dye capable of staining erythroblasts but not leukocytes and the detection 10 of scattered light at two different angles and fluorescent light by a flowcytometer.

In these methods, however, cell membranes of leukocytes as well as erythroblasts become easily damaged especially in a hematologic sample with increasing time after collection of blood.

15 Accordingly, some leukocytes are stained with the dye that on purpose to stain erythroblasts. As a result, erythroblasts cannot be accurately determined. For example, in the case where scattered light and fluorescent light are detected, a site where erythroblasts appear overlaps or overlays a site where leukocytes appear. Especially, when 20 lymphoid cells are damaged, it becomes more difficult to distinguish erythroblasts from damaged lymphoid cells clearly and thus the presence of erythroblasts cannot be detected accurately.

Moreover, in recent years, more and more medical laboratories send blood samples taken from patients to institutions 25 specialized for mass examination in order to reduce costs and improve

efficiency. In such a case, it sometimes takes one day or more from the collection of blood to the examination.

Besides, it is difficult to accurately discriminate and count erythroblasts in a sample containing lymphoblasts or a sample in which chemotherapy or the like has made membranes of leukocytic cells ready to be damaged by an hemolytic agent, even if the sample does not go through change with time.

Additionally, Japanese Patent Publication No. HEI 8(1996)-1434 discloses a method for identifying nucleated red blood cells and the like. This method includes the addition of thiazole orange to a blood sample, the addition of two kinds of fluorescent labeled antibodies, anti-CD45 and anti-CD71, and the detection of signals at at least three fluorescent channels and at least two light scattering channels by a flowcytometer.

This method uses two antibodies and one fluorescent dye as reagents and has a disadvantage in that the reagents are expensive. Since the method can examine immature, nucleated red blood cells only with the combination of these specific antibodies with the specific dye, it is impossible to analyze erythroblasts cheaply by use of this method.

Further, Japanese Unexamined Patent Publication No. HEI 2(1990)-73157 discloses a method for analyzing various kinds of cells including nucleated red blood cells by detecting signals at at least three fluorescent channels and at least two light scattering channels by a flowcytometer using two kinds of fluorescent nucleic acid dyes

and a fluorescent monoclonal antibody.

According to this method, in order to discriminate erythroblasts from leukocytes, a blood sample is stained with the fluorescent monoclonal antibody, and side scattered light is measured.

5 However, since this publication lacks description about the distinction of erythroblasts from platelets and debris, it is difficult to count erythroblasts exactly by this method.

Japanese Patent No. 2620810 discloses a method for detecting fluorescent light and scattered light by a flowcytometer.

10 The method includes the lysis of erythrocytes and the addition of a monoclonal antibody, a fixative and a nucleic acid dye which binds with DNA first.

According to this method, since a sample must be first subjected to erythrolytic treatment, the sample must be subjected to 15 centrifugal cleaning immediately after the erythrolytic treatment. Thus absolute counting is difficult. Moreover, since this centrifugal cleaning involves complicated operation, results of detection vary significantly depending on the skill of an examiner.

Under these circumstances, there has been a demand for an 20 easy, inexpensive method for analyzing erythroblasts accurately even in a hematologic sample collected some time before. There has also been a demand for a method for classifying and counting erythroblasts according to a degree of maturity thereof. However, such method has not been established so far.

SUMMARY OF THE INVENTION

The present invention provides a method for discriminating and counting and staging erythroblasts comprising the steps of:

- (i) staining leukocytes in a hematologic sample by adding a fluorescent labeled antibody capable of binding specifically with leukocytes to the hematologic sample;
- (ii) raising the permeability only of cell membranes of erythroblasts in the hematologic sample to a nucleotide fluorescent dye which does not permeate a cell membrane usually;
- 10 (iii) staining nuclei of the erythroblasts in the hematologic sample with the nucleotide fluorescent dye;
- (iv) subjecting the hematologic sample to flowcytometry to detect at least two fluorescent signals from each cell; and
- (v) discriminating and counting the erythroblasts from 15 difference in intensity between the at least two fluorescent signals.

The present invention is now described in detail by way of embodiments, which are not intended to limit the scope of the invention, with reference to the accompanying drawings in which:

20 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a scattergram obtained by the method for discriminating and counting erythroblasts in accordance with the present invention;

Fig. 2 is a schematic view of Fig. 1;

25 Fig. 3 is a graph showing correlation between the ratio of

erythroblasts to leukocytes counted by the method of the present invention and that counted by a manual microscopic method;

5 Figs. 4A to 4C show changes with time in scattergrams obtained by the method for discriminating and counting erythroblasts in accordance with the present invention;

Figs. 5A to 5C show changes with time in scattergrams in another sample obtained by the method for discriminating and counting erythroblasts in accordance with the present invention;

10 Fig. 6 is a scattergram in the case where scattered light signals are combined with the method for discriminating and counting erythroblasts in accordance with the present invention;

Fig. 7 is a scattergram in which erythroblasts are distinguished from ghosts in a group of ghosts and nucleated red blood cells in Fig. 6;

15 Fig. 8 is a scattergram showing distribution of individual cells with intensity of green fluorescence and that of red fluorescence as coordinate axes used for NRBC staging;

Fig. 9 illustrates enclosure of each group of blood cells with windows for analysis;

20 Fig. 10 is a graph showing correlation between the ratio of Stage II to total erythroblast counted by the method of the present invention and that counted by a manual microscopic method;

25 Fig. 11 is a graph showing correlation between the ratio of State III to total erythroblast counted by the method of the present invention and that counted by a manual microscopic method.

DETAILED DESCRIPTION OF THE INVENTION

The hematologic sample used in step (i) according to the present invention is a sample of body fluid containing leukocytes and erythroblasts, such as a sample of peripheral blood, bone marrow or 5 lymphoid tissue or urine, or a sample taken by apheresis.

Fluorescent labeled antibodies capable of binding specifically with leukocytes such as anti-CD45 antibody. A commercially available antibody product may be employed.

Fluorescent labeling compounds for converting the above-10 mentioned antibodies to the fluorescent labeled antibodies include phycoerythrin, fluorescein isothiocyanate (FITC), allophycocyanin, Texas Red, CY5, and a peridinin chlorophyll complex, which may be used singly or as a combination thereof. Preferably, these fluorescent labeling compounds have a fluorescent spectrum different from that of 15 the fluorescent dye specific to nucleotide explained later.

Phycoerythrin and fluorescein isothiocyanate are preferred among the above compounds.

The mixture ratio of the hematologic sample to the 20 fluorescent labeled antibody may suitably be changed depending on the state of the hematologic sample, the kind of the fluorescent labeled antibody and the like, but the mixture ratio may be about 10 : 1 to 2 : 1 by volume, for example. The mixing of the hematologic sample with the fluorescent labeled antibody may be carried out for a time period suitably set depending on temperature, but preferably at room 25 temperature for 15 to 30 minutes or in an ice bath for 30 to 45 minutes,

for example.

In step (ii) of the present invention, the permeability only of cell membranes of erythroblasts to the nucleotide fluorescent dye is raised. Usually, the nucleotide fluorescent dye does not penetrate cell
5 membranes.

Examples of the nucleotide fluorescent dyes are propidium iodide, N-methyl-4-(1-pyrene)vinyl-propidium iodide, ethidium bromide, TOTO-1, TOTO-3, YOYO-1, YOYO-3, BOBO-1, BOBO-3, ethidium homodimer-1 (EthD-1), ethidium homodimer-2 (EthD-2),
10 POPO-1, POPO-3, BO-PRO-1, YO-PRO-1 and TO-PRO-1, among which propidium iodide is preferred. These nucleotide fluorescent dyes preferably have a fluorescent spectrum different from the fluorescent labeling compound for the fluorescent labeled antibody which binds specifically with leukocytes in step (i), as described above.

15 End concentration of the nucleotide fluorescent dye is about 0.003mg/L to 200mg/L, preferably about 0.03mg/L to 70mg/L, more preferably about 0.3mg/L to 35mg/L. The end concentration here means a concentration in a mixture of the hematologic sample, the fluorescent labeled antibody and the nucleotide fluorescent dye which
20 is subjected to flowcytometry, or in the case where another agent is used as described below, in a mixture just about to be subjected to flowcytometry, preferably.

In order to raise the permeability only of the erythroblasts to the above-mentioned nucleotide fluorescent dye, for example, ① a
25 first reagent fluid of hypotonic osmolarity containing a buffer for

5 maintaining pH within an acidic range is admixed to the hematologic sample, and ② admixed thereto is a second reagent fluid containing a buffer for neutralizing the mixture of the hematologic sample and the first reagent fluid in step ① and adjusting the pH of the mixture to a
10 level suitable for staining and an osmolarity compensating agent for adjusting the osmolarity of the mixture within such a range as retains the shape and integrity of leukocytes.

10 The first reagent fluid in step ① is maintained at a pH within the acidic range, for example, from about 2.0 to 5.0, preferably from about 2.5 to 4.0, and more preferably from about 3.0 to 3.5, for effective lysis of erythrocytes. If the pH is too low, the reagent fluid does excessive damage to not only erythrocytes but also leukocytes, erythroblasts and the fluorescent labeled antibody specific to leukocytes. If the pH is too high, the action of the reagent fluid lysing erythrocytes is restrained.
15

20 The buffer to maintain the pH within the above-mentioned range is a buffer having an acid dissociation constant pKa of about 3.0 ± 2.0. Examples of such buffers are malic acid and its salt, succinic acid and its salt, citric acid and its salt, phosphoric acid and its salt, and Good's buffer. The concentration of the buffer is not particularly limited so long as it is enough for maintaining the pH of the first reagent fluid at about 2.0 to 5.0. For example, the concentration may be 5 to 50 mM/l.

25 The first reagent fluid is required to have a hypotonic osmolarity, for example, an osmotic pressure not exceeding about 100

mOsm/kg•H₂O, more preferably from about 10 to 60 mOsm/kg•H₂O. The kind of osmolarity compensating agent for adjusting the first reagent fluid within the above range of osmotic pressure is not particularly limited, but examples thereof are alkali metal salts and 5 saccharides. For example, sodium chloride or sucrose may be used in a concentration of about 0.1g/L to 2.0g/L, for example. However, in the case where the first reagent fluid can compensate the above osmotic pressure by itself, it may not be necessary to use the osmolarity compensating agent.

10 The hematologic sample is treated with the first reagent fluid for a time period necessary for complete lysis of erythrocytes, for example, for about 5 to 120 seconds, preferably for about 10 to 60 seconds, more preferably for about 20 to 40 seconds.

15 The mixture ratio of the hematologic sample to the first reagent fluid is not particularly limited. However, in consideration of operation by a flowcytometer, the mixture ratio may be about 1 : 5 to 1 : 200 by volume, for example.

20 The second reagent fluid in step ② contains the buffer to neutralize the mixture of the hematologic sample and the first reagent fluid and adjust the mixture to a pH suitable for staining and the osmolarity compensating agent to adjust the mixture to an osmotic pressure suitable for retaining the shape and integrity of leukocytes.

25 The pH of the second reagent fluid which neutralizes acidity of the first reagent fluid and is suitable for staining is, for example, about pH5.0 to pH11.0, preferably about pH7.5 to pH10.0. The kind

of a buffer used for maintaining the pH within these ranges is not particularly limited, but a buffer having a pKa of about 9.0 ± 2.0 is preferred. Examples thereof are phosphoric acid and its salt, HEPES, Tricine, and the like. The concentration of the buffer is not 5 particularly limited so far as it can maintain the second reagent fluid at a pH about 5.0 to 11.0, for example, 5 to 100 mM/l.

The osmotic pressure of the second reagent fluid suitable for retaining the shape and integrity of leukocytes is about 300 mOsm/kg•H₂O to 1000 mOsm/kg•H₂O, more preferably 400 10 mOsm/Kg•H₂O to 600 mOsm/kg•H₂O. The kind of an osmolarity compensating agent used for adjusting the osmotic pressure within the above ranges is not particularly limited. Examples thereof are alkali metal salts and saccharides. For example, sodium chloride or sucrose may be used in a concentration of about 10.0g/L to 20.0g/L, 15 for example.

The mixture ratio of the first reagent fluid to the second reagent fluid can be suitably adjusted depending on the pH and amount of the first reagent fluid used formerly, the concentration of the osmolarity compensating agent in the first reagent fluid, and the 20 pH of the second reagent fluid, the concentration of the osmolarity compensating agent in the second reagent fluid and the like. For example, in the case where the pH and the osmotic pressure of the first reagent fluid are 3.0 and 16 mOsm/kg•H₂O, respectively, and the pH and the osmotic pressure of the second reagent fluid are 7.5 and 400 25 mOsm/kg•H₂O, respectively, the mixture ratio of the first reagent fluid

to the second reagent fluid is preferably about 1 : 1 to 1 : 5.

For retaining the shape and integrity of leukocytes in the hematologic sample in step (ii) of the present invention, the osmotic pressure after the addition of the first reagent fluid and the second reagent fluid is preferably within the range from about 100 to 500 mOsm/kg•H₂O, more preferably from 200 to 400 mOsm/kg•H₂O. If the osmotic pressure after the addition of the first reagent fluid and the second reagent fluid is out of this range, it is preferable that the second reagent fluid contains an osmolarity compensating agent. The kind of the osmolarity compensating agent is not particularly limited, but is preferably a substance capable of maintaining normal biological cells at physiologic osmotic pressure, such as an alkali metal or a saccharide.

In step (iii) of the present invention, the nuclei of the erythroblasts are stained. For staining the nuclei of the erythroblasts, the hematologic sample treated in the above-described steps is stained with the nucleotide fluorescent dye. More particularly, the nucleotide fluorescent dye may be added to the first reagent fluid or second reagent fluid beforehand, and this first reagent fluid or second reagent fluid containing the nucleotide fluorescent dye may be mixed with the hematologic sample. Alternatively, a reagent fluid containing the nucleotide fluorescent dye may be separately added. The timing of adding the reagent fluid containing the nucleotide fluorescent dye is not limited, but it is preferred that the nucleotide dye is added into the first reagent fluid beforehand. A time required for the staining of the

nuclei of the erythroblasts is about 1 to 120 minutes, preferably in about 3 to 30 minutes, more preferably in about 5 to 10 minutes, after the hematologic sample is mixed with all the reagents.

The flowcytometer used in step (iv) of the present invention is 5 not particularly limited, and may be a commercially available one. By use of such a flowcytometer, at least two fluorescent signals are detected for each cell. The fluorescent signals to be detected depend on what kind of fluorescent labeling compound is used for the fluorescent labeled antibody and what kind of nucleotide fluorescent 10 dye is used. The fluorescent signals may be of a combination of red and green fluorescence, a combination of red and orange fluorescence, and a combination of green and orange fluorescence, among which the combination of red and green fluorescence is preferred.

In step (v) of the present invention, erythroblasts are 15 discriminated and counted based on difference in intensity between the above-mentioned at least two fluorescent signals. For example, in the case where two fluorescent signals are detected, a two-dimensional distribution chart (scattergram) is preferably produced by plotting the fluorescence based on the fluorescent labeled antibody which binds 20 specifically with leukocytes and the fluorescence based on the nucleotide fluorescent dye in two axes. An area in which erythroblasts appear may be defined on this two-dimensional distribution chart. The erythroblast count can be obtained by counting the number of cells in this area. An area in which 25 leukocytes appear as well as the area in which erythroblasts appear

may be defined on this two-dimensional distribution chart. The number of cells is counted in the individual areas to obtain the erythroblast count and leukocyte count. The erythroblast count is divided by the leukocyte count and thereby the ratio of erythroblasts to 5 leukocytes can be obtained. Alternatively a ratio between erythroblasts and total number of nucleated cell, i.e., erythroblast and leukocyte count combined, may be obtained.

Additionally, in the case where membranes of leukocytes are excessively damaged by effect of added agents and leukocytes cannot 10 be clearly distinguished from erythroblasts in the two-dimensional distribution chart on which the nucleotide fluorescence (red fluorescence in the examples) and the fluorescence based on the fluorescent labeled antibody (green fluorescence in the examples) are plotted in the two axes, the following two steps may further be added in 15 step (iv) for the purpose of discriminating and counting erythroblasts more accurately:

(a) Scattered light signals are also detected, and the scattered light (e.g., side scattered light signals, forward scattered light and the like, preferably side scattered light) and the fluorescence based on the 20 fluorescent labeled antibody are plotted on two axes to produce a two-dimensional distribution chart. From this distribution chart, the group of leukocytes is identified. (see Fig. 6.)

(b) In a distribution chart on which the nucleotide fluorescence and the fluorescence based on the fluorescent labeled antibody are plotted in the two axes, an area in which the 25

corresponding group of leukocytes is distributed is defined. (see Fig. 7.)

(c) A boundary A between the group of leukocytes and a group of erythroblasts is marked in the two-dimensional distribution chart of 5 (b).

Further, in the case where the end concentration of the nucleotide fluorescent dye is 0.003mg/L to 10mg/L in step (ii), the population of erythroblasts can be classified into at least two groups according to the degree of maturity of the erythroblasts which is judged 10 from difference basis of intensity of fluorescent light caused by the nucleotide fluorescent dye. More preferably, a suitable end concentration of the nucleotide fluorescent dye is 0.03mg/L to 3mg/L.

In this case, in step (v), it is possible to measure the 15 maturation and count erythroblasts in different maturation stages from difference in intensity of fluorescence based on the nucleotide fluorescent dye, in other words, it is possible to determine an area where erythroblasts are present in the scattergram; determine sub-areas of groups of erythroblasts in different stages of maturity in the 20 determined erythroblast area from difference in intensity of fluorescent light caused by the nucleotide fluorescent dye; count the number of cells in each of the sub-areas; divide the number of erythroblasts in each of the stages of maturity by the number of all the erythroblasts to obtain a ratio of erythroblasts in each of the stages of maturity to the whole erythroblasts.

25 The classification of erythroblasts according to their degrees

of maturity means classifying morphologic proerythroblast basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatic erythroblast or the like in erythroblasts into at least two groups or into the respective groups. For example, erythroblasts are classified into 5 proerythroblasts and three groups of basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatic erythroblasts, in Example 3.

EXAMPLE

10 Examples of the method for discriminating and counting erythroblasts are now described.

Example 1

First, reagents having the following compositions were prepared.

15 Fluorescent labeled antibody:

FITC labeled anti-CD45 antibody

First reagent fluid (pH 3.0, osmotic pressure 16 mOsm/kg•H₂O):

Buffering agent - Citric acid monohydrate, 2.10 g/l

- Disodium hydrogenphosphate, 0.56 g/l

20 Nucleotide fluorescent dye

- Propidium iodide, 100 mg/l

Purified water

Second reagent fluid (pH 7.5, osmotic pressure 420 mOsm/kg•H₂O):

Buffering agent - Sodium dihydrogenphosphate dihydrate,

25 0.95 g/l

- Disodium hydrogenphosphate, 6.24 g/l

Osmolarity compensating agent

- Sodium chloride, 10.2g/l

Purified water

5 Fifty (50) μ l of blood from a patient was added to peripheral blood to produce a hematologic sample. These bloods had been treated with an anticoagulant. Ten (10) μ l of the above FITC labeled anti-CD45 antibody were added to the hematologic sample. This mixture was incubated at room temperature for about 15 minutes.

10 Here, the blood containing erythroblasts and the peripheral blood were used within eight hours after they were collected.

Then, 500 μ l of the first reagent fluid were added to the mixture, which was incubated at room temperature for about 30 seconds. To the resulting mixture, 1000 μ l of the second reagent fluid were added and then the mixture was incubated at room temperature for about 5 minutes. Then, fluorescences of wavelengths of 530nm (green) and 650nm (red) were detected for individual cells contained in the obtained hematologic sample by a flowcytometer equipped with an argon ion laser of 488nm wavelength as a light source.

20 Fig. 1 is a scattergram showing distribution of the cells in which the intensity of green fluorescence and that of red fluorescence are plotted in coordinate axes.

In Fig. 1, there were observed four groups: leukocytes, red fluorescent-stained leukocytes, erythroblasts and ghosts.

25 Analysis was made as follows: First the leukocytes and red

fluorescent-stained leukocytes were enclosed in a window (W1) as shown in Fig. 2, and all counted to obtain the leukocyte count. Next, all erythroblasts were enclosed by another window (W2) and were counted to obtain the erythroblast count. The obtained erythroblast 5 count was divided by the obtained leukocyte count to obtain the ratio of the erythroblasts to the leukocytes.

Separately from the above example, the same hematologic sample as in Example 1 was subjected to a manual microscopic method (May Grünwald-Giemsa stain, 1,000 WBC count) 10 discrimination and counting to obtain the morphologic erythroblast count.

Fig. 3 shows a correlation between the ratio of erythroblast determined by the flowcytometer in accordance with the present invention and that determined by the manual microscopic method.

15 From Fig. 3, correlation coefficient r is 0.991, which shows that the method of the present invention is remarkably accurate in discriminating and counting erythroblasts.

Example 2

Blood containing erythroblasts from two patients other than 20 the patient in Example 1 and peripheral blood (preserved at room temperature for 8 hours, 24 hours, 48 hours after collection) were examined about leukocytes and erythroblasts in the same manner as in Example 1. The results are shown in Figs. 4A to 4C and Figs. 5A to 5C.

25 The ratio of the erythroblasts to the leukocytes was also

calculated for each of the samples. The results are shown in Table 1.

Table 1

	after 8 hours	after 24 hours	after 48 hours
Sample 1	3.6%	3.6%	3.6%
Sample 2	6.1%	6.1%	6.7%

5 Figs. 4A to 4C and 5A to 5C and Table 1 show that the passing of time hardly affects the results of examination according to the present invention.

Example 3

Reagents having the following compositions were prepared.

10 Fluorescent labeled antibody:

FITC labeled anti-CD45 antibody

First reagent fluid (pH 3.0, osmotic pressure 16 mOsm/kg):

Buffering agent - Citric acid monohydrate, 2.10 g/l

- Disodium hydrogenphosphate, 0.56 g/l

15 Nucleotide fluorescent dye

- Propidium iodide, 1 mg/l

Purified water

Second reagent fluid (pH 7.5, osmotic pressure 420 mOsm/kg):

Buffering agent - Sodium dihydrogenphosphate

20 dihydrate, 0.95 g/l

- Disodium hydrogenphosphate, 6.24 g/l

Osmolarity compensating agent

- Sodium chloride, 10.2g/l

Purified water

Fifty (50) μ l of blood from a patient was mixed with ten (10) μ l of the above FITC labeled anti-CD45 antibody. These bloods had been 5 treated with an anticoagulant. This mixture was incubated at room temperature for about 15 minutes.

Then, 500 μ l of the first reagent fluid were added to the mixture, which was incubated at room temperature for about 30 seconds. To the resulting mixture, 1000 μ l of the second reagent fluid 10 were added and then the mixture was incubated at room temperature for about 5 minutes. Then, fluorescences of wavelengths of 530nm (green) and 650nm (red) were detected about individual cells contained in the obtained hematologic sample by a flowcytometer equipped with an argon ion laser of 488nm wavelength as a light source.

15 Fig. 8 is a scattergram showing distribution of the cells in which the intensity of green fluorescence and that of red fluorescence are plotted in coordinate axes.

In Fig. 8, there were observed six groups: leukocytes, red 20 fluorescent-stained leukocytes, mature erythroblasts, immature erythroblasts 1, immature erythroblasts 2 and ghosts.

Analysis was made as follows: First the leukocytes and red fluorescent-stained leukocytes were enclosed in a window (W1) as shown in Fig. 9, and all counted to obtain the leukocyte count. Next, all erythroblasts were enclosed by another window (W2) and were 25 counted to obtain the total erythroblast count. Then Stage I, Stage II

and Stage III in the window (W2) were enclosed by windows (W3), (W4) and (W5), respectively. The erythroblasts in the respective windows were counted to obtain the erythroblast staging count. The erythroblast number in the individual window was divided by the total 5 erythroblast number to obtain the proportion of erythroblast in different stages.

Separately from the above example, the same hematologic sample as in Example 3 was subjected to a manual microscopic method (May Grünwald-Giemsa stain) discrimination and counting to 10 classify morphologically erythroblasts into proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatic erythroblasts and count the numbers thereof.

Table 2 shows results determined by the flowcytometer in accordance with the present invention and results determined by the 15 manual microscopic method.

Table 2

Method of the Invention		Manual Microscopic Method	
Stage I	0.7%	Proerythroblast plus Basophilic erythroblast	0%
Stage II	17.6%	Polychromatophilic Erythroblasts	18%
Stage III	81.7%	Orthochromatic Erythroblast	82.0%

Table 2 shows that the results by the method the present 20 invention agrees well with the results by the manual microscopic

method.

Example 4

Twenty-four (24) samples of bloods form patients were determined by the method of the above Example 3. Correlation 5 between the method of the invention and the manual microscopic method was analyzed. Results were shown in Figs 10 and 11. Figs. 10 and 11 shown that the method of the invention has a definite correlation with the manual microscopic method and is capable of classifying and counting erythroblasts in the different maturity stages 10 with great accuracy.

WHAT IS CLAIMED IS:

1. A method for discriminating and counting erythroblasts comprising the steps of:

5 (i) staining leukocytes in a hematologic sample by adding a fluorescent labeled antibody capable of binding specifically with leukocytes to the hematologic sample;

(ii) raising the permeability only of cell membranes of erythroblasts in the hematologic sample to a nucleotide fluorescent 10 dye which does not permeate a cell membrane usually, the nucleotide fluorescent dye having a fluorescent spectrum capable of being distinguished from that of a fluorescent labeling compound of the fluorescent labeled antibody in step (i);

15 (iii) staining nuclei of the erythroblasts in the hematologic sample with the nucleotide fluorescent dye;

(iv) subjecting the hematologic sample to flowcytometry to detect at least two fluorescent signals from each cell; and

(v) discriminating and counting the erythroblasts from difference in intensity between the at least two fluorescent signals.

20

2. A method according to claim 1, wherein the fluorescent labeled antibody capable of binding specifically with leukocytes in the step (i) recognizes an antigen present on leukocytes surface and binds with the antigen.

3. A method according to claim 1 or 2, wherein the fluorescent labeling compound of the fluorescent labeled antibody in the step (i) comprises at least one compound selected from the group consisting of phycoerythrin, fluorescein isothiocyanate, allophycocyanin, Texas Red, 5 CY5, a peridinin chlorophyll complex, and a combination thereof.

4. A method according to claim 1, wherein the raising of the permeability only of cell membranes of erythroblasts in the hematologic sample to the nucleotide fluorescent dye in step (ii) 10 comprises the steps of:

① admixing a first reagent fluid of hypotonic osmolarity containing a buffer for maintaining pH within an acidic range to the hematologic sample after the step (i); and

② admixing thereto a second reagent fluid containing a buffer for neutralizing the first reagent fluid containing the hematologic sample and adjusting a mixture of the hematologic sample and the first reagent fluid to a pH suitable for staining and an osmolarity compensating agent for adjusting the mixture to an osmolarity suitable for retaining the shape and integrity of leukocytes.

20 5. A method according to any of claim 1 or 4, wherein the staining of the nuclei of the erythroblasts in the step (iii) is carried out by mixing the hematologic sample with the nucleotide fluorescent dye.

25 6. A method of claim 5, wherein the nucleotide fluorescent dye

comprises at least one compound selected from the group consisting of propidium iodide, N-methyl-4-(1-pyrene)-vinyl-propidium iodide, ethidium bromide, TOTO-1, TOTO-3, YOYO-1, YOYO-3, BOBO-1, BOBO-3, ethidium homodimer-1, ethidium homodimer-2, POPO-1, 5 POPO-3, BO-PRO-1, YO-PRO-1 and TO-PRO-1.

7. A method according to claim 1, wherein the at least two fluorescent signals detected for each cell includes a fluorescent signal based on the fluorescent labeled antibody capable of binding 10 specifically with leukocytes and a fluorescent signal based on the nucleotide fluorescent dye and the two fluorescent signals are plotted in two coordinate axes to obtain a two-dimensional distribution chart.

8. A method according to any of claims 1 to 7, wherein an area in 15 which erythroblasts appear is defined on the two-dimensional distribution chart and the number of cells in the area is counted.

9. A method according to any of claims 1 to 7, wherein areas in which leukocytes and erythroblasts appear are defined on the two- 20 dimensional distribution chart, the number of cells in each of the areas is counted to obtain a leukocyte count and an erythroblast count, and the erythroblast count is divided by the leukocyte count, whereby the ratio of erythroblasts to leukocytes is obtained.

25 10. A method according to claim 5, wherein the nucleotide

fluorescent dye is used at a concentration within the range of 0.003mg/L to 10mg/L in a mixture to be subjected to flowcytometry to stain erythroblasts according to degrees of maturity of the erythroblasts, and thereby the erythroblasts are classified into at least 5 two groups according to the degrees of maturity thereof.

11. A method according to claim 10, wherein:

(1) the at least two fluorescent signals detected for each cell includes a fluorescent signal based on the fluorescent labeled antibody 10 capable of binding specifically with leukocytes and a fluorescent signal based on the nucleotide fluorescent dye and the two fluorescent signals are plotted in two coordinate axes to obtain a two-dimensional distribution chart;

(2) areas are set in the two-dimensional distribution chart for 15 classifying erythroblasts into at least two groups from difference in intensity of the fluorescent signals based on the nucleotide fluorescent dye; and

the number of cells in each of the areas is counted for obtaining counts of erythroblasts at different degrees of maturity.

20

12. A method according to claim 11, wherein an area of all erythroblasts and areas of at least two groups of erythroblasts at different degrees of maturity are defined in the two-dimensional distribution chart, the number of cells in each of the areas is counted 25 to obtain an total erythroblast count and counts of erythroblasts at the

respective degrees of maturity, and the counts of erythroblast at the respective degrees of maturity are divided by the total erythroblast count, whereby the ratios of the erythroblasts at the respective degrees of maturity to all the erythroblasts.

ABSTRACT OF THE DISCLOSURE

A method for discriminating and counting erythroblasts comprises the steps of: (i) staining leukocytes in a hematologic sample by adding a fluorescent labeled antibody capable of binding specifically with leukocytes to the hematologic sample; (ii) raising the permeability only of cell membranes of erythroblasts in the hematologic sample to a nucleotide fluorescent dye which does not permeate a cell membrane usually, the nucleotide fluorescent dye having a fluorescent spectrum capable of being distinguished from that of a fluorescent labeling compound of the fluorescent labeled antibody in step (i); (iii) staining nuclei of the erythroblasts in the hematologic sample with the nucleotide fluorescent dye; (iv) subjecting the hematologic sample to flowcytometry to detect at least two fluorescent signals from each cell; and (v) discriminating and counting the erythroblasts from difference in intensity between the at least two fluorescent signals.

DECLARATION AND POWER OF ATTORNEY
FOR UTILITY PATENT APPLICATION - JOINT

As the below named inventors, we hereby declare that:

Our residences, post office addresses, and citizenships are as stated below next to our names.

We believe that we are the original, first, and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled "PROCESS FOR DISCRIMINATING AND COUNTING ERYTHROBLASTS," the specification of which is attached hereto, this application being a continuation-in-part of application Serial No. 09/019,932 filed Feb. 6, 1998.

We hereby state that we reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information that is material to the examination of this application in accordance with 37 Code of Federal Regulations § 1.56.

We hereby claim foreign priority benefits under 35 United States Code §§ 119 and 365 of any foreign application(s) for the patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Applications: _____

We hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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FIG.1

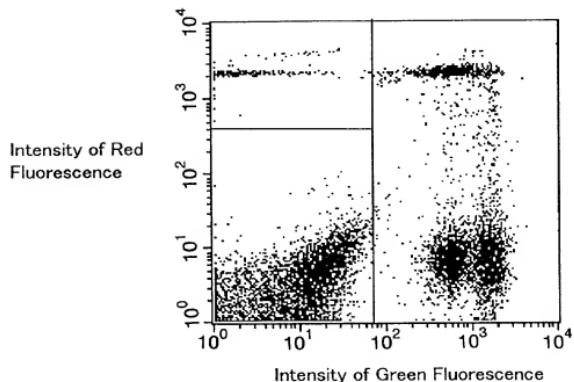


FIG. 2

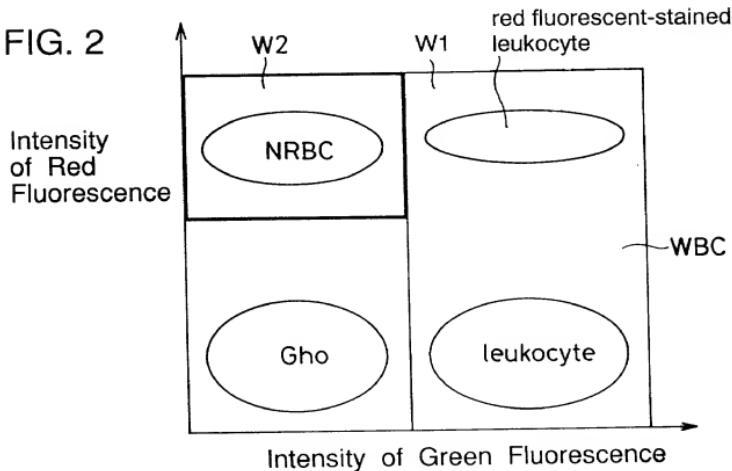


FIG. 3

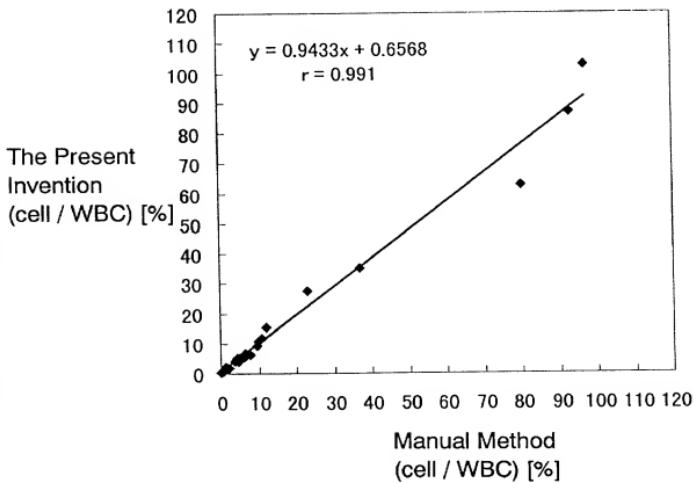


FIG. 4A

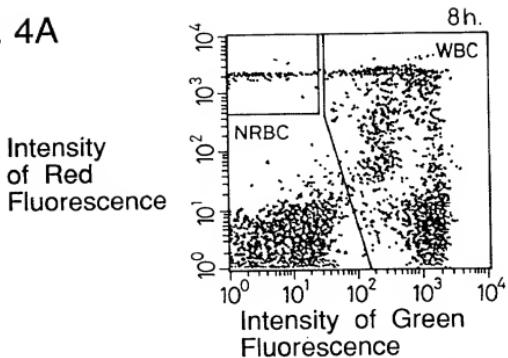


FIG. 4B

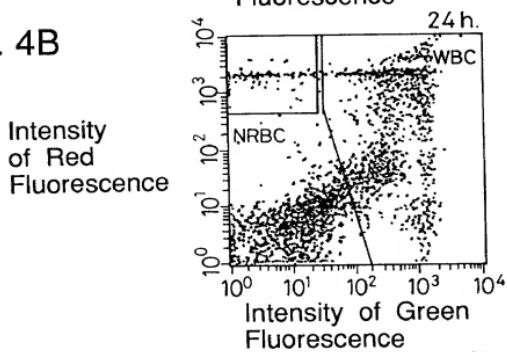


FIG. 4C

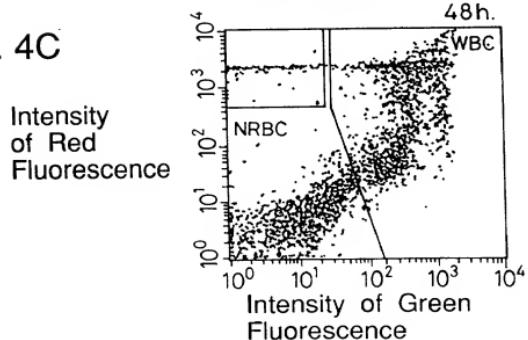


FIG. 5A

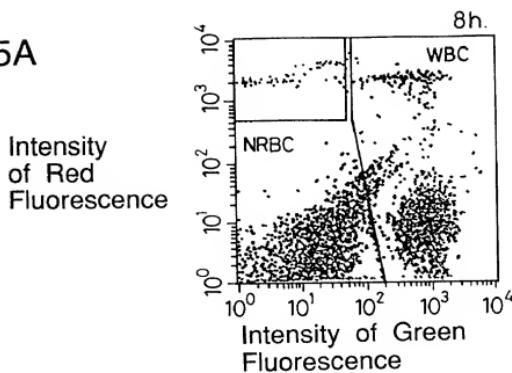


FIG. 5B

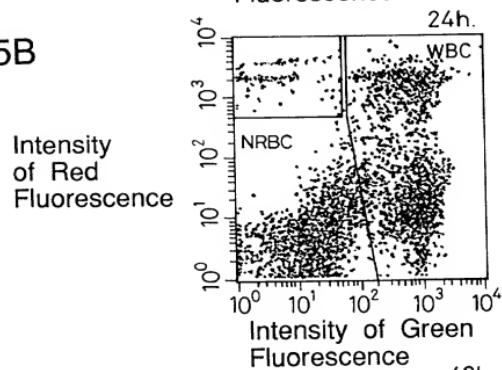


FIG. 5C

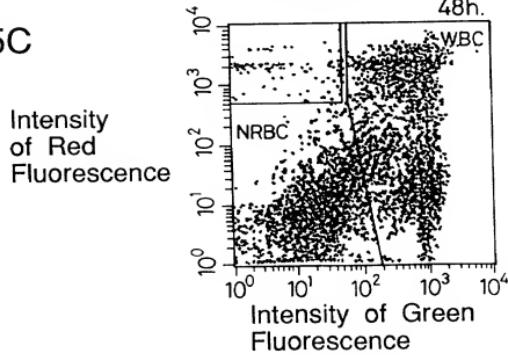
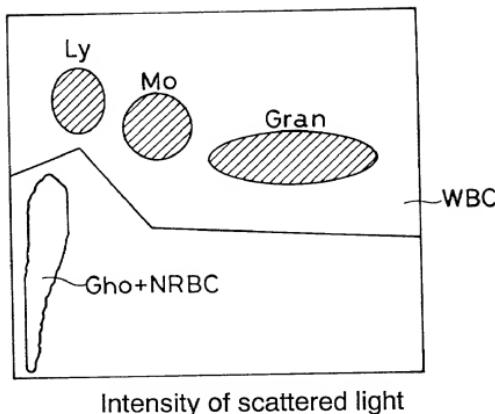


FIG. 6

Intensity of
Green
Fluorescence



Ly : lymphocyte
Mo : monocyte
Gran : granulocyte
WBC : total leukocyte
Gho : ghost
NRBC : erythroblast

FIG. 7

Intensity of Red
Fluorescence

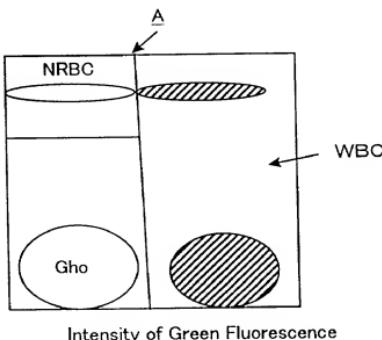


FIG.8

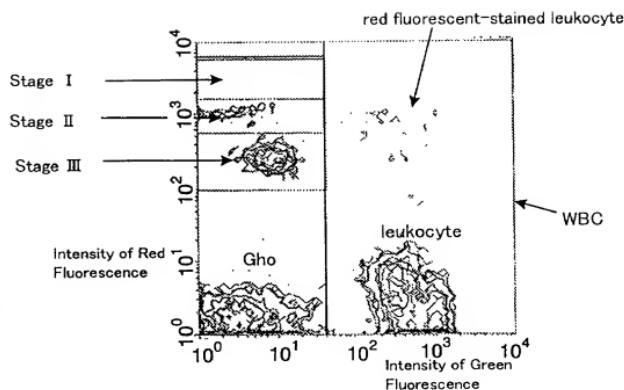


FIG.9

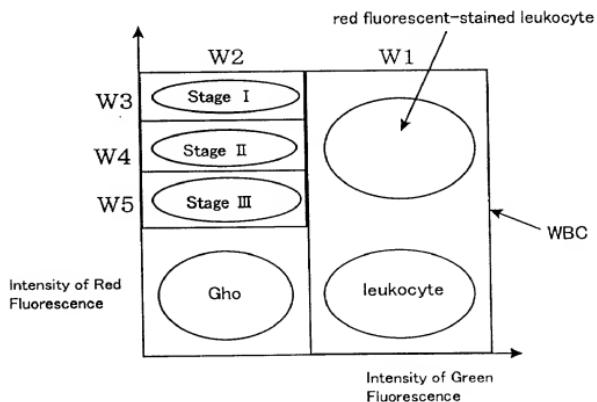


FIG.10

Stage II

The Present Invention
(STAGE II /NRBC)[%]

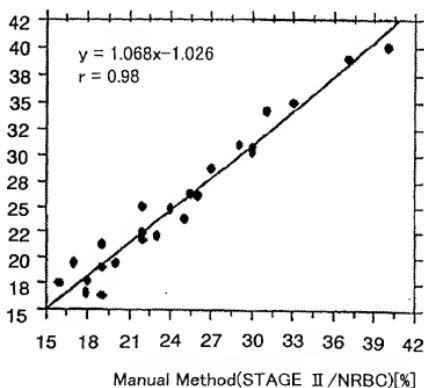


FIG.11

Stage III

The Present Invention
(STAGE III /NRBC)[%]

